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Development of a Transgenic *Flammulina velutipes* Oral Vaccine for Hepatitis B

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SUMMARY

Orally administered fungal vaccines show promise for the prevention of infectious diseases. Edible mushrooms are deemed appropriate hosts to produce oral vaccines due to their low production cost and low risk of gene contamination. However, their low expression level of antigens has limited the potential development of oral vaccines using mushrooms. The low expression level might result from impurity of the transgenic mycelia since dikaryotic mycelia are commonly used as transformation materials. In this study, stable transgenic hepatitis B virus surface antigen (HBsAg) in *Flammulina velutipes* transformants was obtained by *Agrobacterium*-mediated transformation, followed by fruiting and basidiospore mating. The formation of HBsAg was detected by western blot analysis. The expression levels of HBsAg in transgenic *F. velutipes* fruiting bodies were (129.3±15.1), (110.9±1.7) and (161.1±8.5) ng/g total soluble protein. However, the values may be underestimated due to incomplete protein extraction. Two of the four pigs in the experimental group produced positive anti-HBsAg-specific IgG after being fed the HBsAg transgenic *F. velutipes* fruiting bodies for 20 weeks, while no anti-HBsAg antibody was detected in the control group. One of the positive pigs had HBsAg titres of 5.36 and 14.9 mIU/mL in weeks 10 and 14, respectively, but expression faded thereafter. The other positive pig displayed HBsAg titres of 9.75, 17.86 and 39.87 mIU/mL in weeks 14, 18 and 20, respectively. The successful immunogenicity in pigs fed transgenic *F. velutipes* fruiting bodies demonstrated the potential of using the fungus as an oral vaccine.

Key words: *Agrobacterium*-mediated transformation, *Flammulina velutipes*, hepatitis B, mating, oral vaccine

INTRODUCTION

Although vaccinations have saved millions of lives from infectious diseases, full implementation of global vaccination remains a challenge due to the relatively high costs of conventional vaccinations incurred by mass production, refrigeration and transportation, as well as by training and compensating personnel for their administration. To overcome these problems, many efforts have been devoted to the development of novel and cost-effective vaccination procedures and technologies. In addition to injected vaccines, oral administration of either raw materials or extracts from transgenic plants, spirulina or yeasts has been reported to stimulate systemic and mucosal immunity (*1*).

Oral vaccines are known to stimulate multiple types of immunity, including mucosal and humoral immunity (*2*). Plant-based oral vaccines were first proposed two decades ago (*3*). The use of transgenic fruits and vegetables for the expression of recombinant protein antigens as oral vaccines has become an attractive topic in plant molecular farming (*1,4*). For example, many efforts have been made to develop hepatitis B oral vaccines by expressing hepatitis B virus surface antigen (HBsAg) in transgenic plants such as tobacco, tomato, potato, banana, tomatillo and rice (*5-12*). In addition to plants, edible mushrooms are also appropriate hosts for the development of oral vaccines (*13*). Using transgenic mushrooms as oral vaccines has all the advantages of a plant-based system coupled with unique benefits, such as complete duplication, fast growth, scaled-up production under controlled

conditions and less gene contamination. A previous study demonstrated the effectiveness of extracts of mycelia derived from edible mushrooms as adjuvants for intranasal influenza vaccines (*14*). Pérez-Martínez *et al.* (*15*) provided a comprehensive review on the use of *Pleurotus* as carrier of oral subunit vaccines. While mushroom-based oral vaccines seem promising, the low expression level of antigens and the instability of the transformants limit the development of mushroom molecular farming. Previously, we demonstrated that heterologous protein expression in the enoki mushroom *Flammulina velutipes* is notably enhanced by using 2A peptide-mediated cleavage to co-express multiple copies of a single gene (*16*). Using this polycistronic expression strategy, enterovirus 71 virus-like particles were successfully produced in transgenic *F. velutipes* (*17*).

Mating and fruiting body formation are sexual stages in the life cycle of basidiomycete fungi and lead to karyogamy, meiosis and basidiospore formation. *F. velutipes* is a heterothallic basidiomycete fungus whose life cycle is characterized by haploid and diploid stages (*18*). *F. velutipes* has a tetrapolar mating type system that generates basidiospores with four possible mating types. After germination, the haploid spores develop into monokaryotic mycelia with AxBx, AxBy, AyBx or AyBy mating types. Although single mating types have been known to produce fruiting bodies under severe stress, most fruiting requires the plasmogamy of two genetically different monokaryotic mycelia, AxBx/AyBy or AxBy/AyBx, to form the dikaryotic mycelia that consist of two nuclei in a cell and the structure of clamp connection (*19*). The generation of transgenic mushrooms usually involves *Agrobacterium*-mediated transformation using modified mycelial pellets, followed by the selection of transformants under appropriate pressures (*17*). Transformants are selected if the foreign gene is inserted into the chromosomal DNA in at least one nucleus of the mycelia. Consequently, the expression level and stability of heterologous genes are reduced if the transformants contain both transgenic and non-transgenic cells. Therefore, it is crucial to obtain transformants containing the transgene in all nuclei.

In this study, stable HBsAg transgenic *F. velutipes* transformants were obtained by *Agrobacterium*-mediated transformation, followed by fruiting and basidiospore mating. We demonstrated that specific immunogenicity was detected in pigs after feeding HBsAg transgenic *F. velutipes* fruiting bodies for a period of time.

MATERIALS AND METHODS

Strains and media

Flammulina velutipes BCRC 930110, a patented strain deposited at the Bioresource Collection and Research Centre (Hsinchu, Taiwan), was grown and maintained on complete yeast medium (CYM) agar or in broth containing 0.2 % tryptone (BD Bioscience, Sparks, MD, USA), 0.2 % yeast extract (Bio Basic, Amherst, NY, USA), 1 % maltose (Sigma-Aldrich, Merck, St. Louis, MO, USA), and 2 % glucose (Bio Basic) at 25 °C. *Escherichia coli* DH5α (Gibco BRL, Life Technologies, Grand Island, NY, USA), which was used for DNA manipulation and plasmid conservation, was grown in Luria-Bertani (LB) medium (Sigma-Aldrich, Merck) at 37 °C. *Agrobacterium tumefaciens* strain LBA4404, kindly provided by Dr Yee-Yung Charng, Agricultural Biotechnology Research Centre, Academia Sinica (Taipei, Taiwan), was used for transformations and was grown in LB medium at 28 °C.

Plasmid construction

A promoter region of the *Agaricus bisporus* glyceraldehyde-3-phosphate dehydrogenase (*Agpd*) gene was amplified from the genome of *A. bisporus* by using primers Agpd-f (5′-TTAAGAGGTCCGCAAGTAGATTGA-3′) and Agpd-r (5′-ATGTGTGTTGTTCGAATAGCGG-3′). The plasmid pGEM-Agpd was constructed by cloning the *Agpd* promoter into a pGEM-T Easy vector (Promega, Madison, WI, USA). The *E. coli* hygromycin B phosphotransferase (*hpt*) gene with cauliflower mosaic virus (CaMV) 35S terminator was amplified from pCAMBIA 1300 (Cambia, Canberra, Australia) using primers *Spe*I-Hpt-f (5´-ACTAGTATGAAAAAGCCTGAACTCACC-3´) and *Pst*I-CaMV 35S terminator-r (5´-TTACACAATAATTCAACAGACGTCGG-3´). Then, the amplified fragment was cloned into a pGEM-Agpd by restriction enzymes *Spe*I and *Pst*I. The backbone plasmid p0390-AH was constructed by introducing the *hpt* and CaMV 35S terminator gene driven by *Agpd* promoter into the pCAMBIA0390 (p0390) vector (CAMBIA) by using restriction enzymes *Nco*I and *Pst*I. The *F. velutipes gpd* (*Fgpd*) promoter was amplified from the *F. velutipes* genome by using primers *Nco*I-Fgpd-f (5′-CCATGGGCATTACTTCGCTCTA-3′) and *Kpn*I-Fgpd-r (5′-CGGTACCTTGTAGATGAGGAG-3′). The plasmid pGEM-Fgpd was constructed by cloning the *Fgpd* promoter into a pGEM-T Easy vector. The codon usage of hepatitis B virus surface antigen (*HBsAg*) derived from plasmid pRc/CMV-HBs(S) (Aldevron, Fargo, ND, USA) was modified according to the codon bias of *F. velutipes*, and an endoplasmic reticulum (ER) retention signal (HDEL) was fused to the C terminus of HBsAg protein to improve its expression. The gene of *HBsAg* was amplified by primers *Kpn*I-HB-f (5′-GGTACCGATGGAGAACATCACAT-3′) and *Bst*EII-HB-r (5′-GGTCACCTTACAGTTCATCATGACTAGCAA-3′) and cloned into a pGEM-Fgpd by restriction enzymes *Kpn*I and *Bst*EII. The construct carrying the gene encoding *HBsAg* (accession number MG717400) driven by the *F. velutipes gpd* promoter was cut from pGEM-Fgpd by restriction enzymes *Nco*I and *Bst*EII inserted into p0390-AH, and the resulting plasmid was designated as p0390-AH-FmHB. A map of the plasmid constructs is shown in **Fig. 1**.

Transformation procedure

Agrobacterium-mediated transformation (AMT) was performed as described by Chen *et al.* (*20*) with minor modifications. The p0390 or p0390-AH or p0390-AH-FmHB constructs were introduced into *A. tumefaciens* by electroporation (ECM 630; BTX, San Diego, CA, USA). The *A. tumefaciens* strains harbouring the target plasmids were cultivated in LB medium

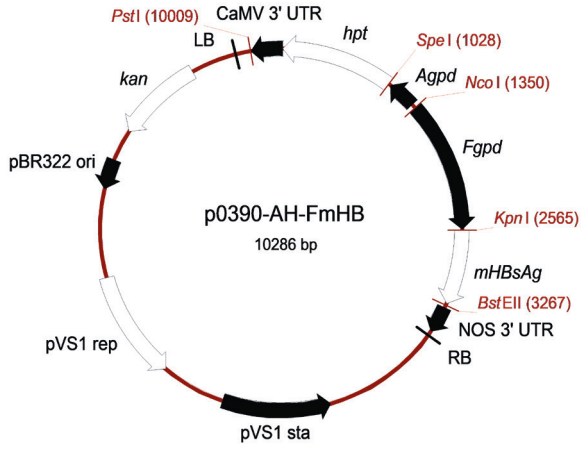

Fig. 1. A map of the plasmid construction: *Agpd*=the *gpd* promoter derived from *Agaricus bisporus*, *Fgpd*=the *gpd* promoter derived from *Flammulina velutipes*, *mHBsAg*=modified hepatitis B virus surface antigen after codon optimization, *kan*=kanamycin-resistant gene from *Escherichia coli, hpt*=hygromycin B phosphotransferase gene derived from *E. coli*, NOS 3′ UTR=nopaline synthase 3′ UTR, CaMV 3′ UTR=cauliflower mosaic virus 35S terminator, pVS1 sta and pVS1 rep=stability and replication regions of vector pVS1, respectively, pBR322 ori=origin of replication of vector pBR322, *Pst*I, *Spe*I, *Nco*I, *Kpn*I and *Bst*EII=restriction enzymes isolated from *Providencia stuartii, Sphaerotilus* sp., *Nocardia corallina, Klebsiella pneumonia* and *Bacillus stearothermophilus*, respectively, LB=left border, RB=right border. Numbers in brackets represent the position of the restriction sites

containing 50 μg/mL of kanamycin (MDBio Inc., Taipei, Taiwan) for 24 h at 28 °C, with shaking at 220 rpm, in an incubator (model S-300R; FIRSTEK, New Taipei City, Taiwan). The strains were then mixed with modified mycelial pellets (MMPs) of *F. velutipes* for 6 h at 23 °C in an induction medium containing 11.8 mM dipotassium phosphate, 10.7 mM monopotassium phosphate, 2 mM magnesium sulfate, 0.6 mM calcium chloride, 9 μM iron(II) sulfate, 43.7 mM 2-(N-morpholino)--ethanesulfonic acid (MES; pH=5.3), 200 μM acetosyringone (all from Sigma-Aldrich, Merck), 2.5 mM sodium chloride, 3.8 mM ammonium sulfate, 0.18 % glucose (all from Bio Basic), and 0.5 % (*m*/*V*) glycerol (Riedel-de Haën; Honeywell, Morris, NJ, USA). After incubation, the MMPs were transferred to fresh induction medium agar plates and incubated at 23 °C for 3-6 days. Then, the treated MMPs were washed with sterile water five times to remove bacteria, transferred to selective agar plates containing 30 μg/mL hygromycin B (Thermo Fisher Scientific, San Jose, CA, USA) and 200 mM cefotaxime (MDBio Inc.) and incubated at 25 °C for 2-3 weeks until hygromycin B-resistant mycelia of *F. velutipes* appeared.

PCR analysis

F. velutipes transformants grown on selective agar plates were subsequently screened *via* PCR analysis to confirm the integration of *HBsAg* gene into the genomes. The *HBsAg* gene fragment was amplified by 96-well thermal cycler (Veriti™ 9902; Applied Biosystems, Foster City, CA, USA) with primers HBsAg-f (5′-GAAGATCTATGGAGAACATCACATCC-3′) and HBsAg-r (5′-GTCGACGGGTCACCTTACAGTTCATCATGA-3′). Genomic DNA was extracted from four-week-old mycelia that were grown in CYM broth with 30 μg/mL hygromycin B, using the CTAB-mini DNA extraction method with minor modifications (*21*). The residual RNA in the isolated DNA was removed by treatment with DNase-free RNase A (GMbiolab Co., Ltd., Taipei, Taiwan).

Mycelium staining

Monokaryotic or dikaryotic mycelia on slides were fixed with 37 % formaldehyde (Sigma-Aldrich, Merck) for 2 min at room temperature. Samples were then rinsed twice with sterile distilled water before incubation for 5 min at room temperature with 1 μg/mL 4',6-diamidino-2-phenylindole (DAPI; Sigma-Aldrich, Merck). The slides were observed by a fluorescence microscope (Eclipse E600; Nikon, Kanagawa, Japan) fitted with a Nikon UV-2A filter (330-380 nm excitation filter, 400 nm dichroic filter, and 420 nm barrier filter).

Fruiting and mating of transformants

The *F. velutipes* transformants were stabilized *via* mating. The fruiting body development procedure was described previously (*22*). Capped polypropylene bottles containing 70 % sawdust and 30 % rice bran were purchased from Wanshen farm (Changhua, Taiwan), autoclaved for 1 h, then inoculated with mycelial plugs and incubated at 23 °C for approx. 3 to 4 weeks. After the vegetative mycelia had grown throughout the substrate, fruiting was induced by water addition, air exposure, and temperature change from 25 to 8 °C. The conditions were kept humid during the fruiting period by watering every other day with sterile distilled water. The basidiospores were collected in a sealed plate (9-cm Petri dishes) from the mature fruiting bodies and separated by resuspending them in sterile water. The suspension was then spread on CYM agar plates containing 30 μg/mL hygromycin B to form monokaryotic mycelia which were verified by DAPI staining. Mating was conducted by placing two mycelial plugs on the same CYM agar plate for 2 weeks at 23 °C. Successful mating was also confirmed by the formation of clamp connections and the presence of two nuclei in a cell. Three dikaryotic transformants were randomly picked for further analysis.

Western blot analysis

For western blot analysis of HBsAg, the fruiting bodies of the *F. velutipes* transformants and of wild-type controls were collected and ground in liquid nitrogen with a mortar and pestle. A total of 40 mg of sample powder was mixed on ice for 1 h with 0.5 mL of protein extraction buffer containing 50 mM potassium phosphate (pH=7.4; Sigma-Aldrich, Merck), 0.1 % Triton X-100 (J.T.Baker, Avantor, Radnor, PA, USA), 300 mM sodium chloride (Bio Basic), and 1 mM phenylmethane sulfonyl fluoride (PMSF; Sigma-Aldrich, Merck). The total soluble protein (TSP) was obtained by centrifugation (13 000×*g*

for 30 min at 4 °C) using Thermo Scientific Sorvall® Legend Mach 1.6 R tabletop centrifuge (Thermo Fisher Scientific), boiled for 20 min with sample buffer containing 50 mM Tris--HCl (pH=7.4; Bio-Rad, Hercules, CA, USA), 2 % sodium dodecyl sulfate (SDS), 0.1 % bromophenol blue, 10 % glycerol, 400 mM dithiothreitol (DTT) and 800 mM 2-mercaptoethanol (all from Sigma-Aldrich, Merck), and separated by 12 % sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). The protein samples were transferred *via* a Trans-Blot® electrophoretic transfer cell (Bio-Rad) to a polyvinylidene difluoride (PVDF) membrane (Millipore, Bedford, MA, USA). Protein was detected using a mouse monoclonal anti-HBsAg antibody (ab9193; Abcam, Taipei, Taiwan) and a goat anti-mouse IgG antibody-conjugated alkaline phosphatase (ab97020; Abcam) in a reaction with 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium (BCIP/NBT) (PerkinElmer, Foster City, CA, USA) as described by the manufacturers.

HBsAg quantification

HBsAg was detected using the SURASE B-96 kit (General Biologicals Corporation, Taipei, Taiwan) according to the manufacturer's instructions. Plate wells coated with anti-HBsAg antibody were incubated with 50 µL of TSP and anti-HBsAg antibody-conjugated peroxidase for 90 min at 37 °C. Each assay was repeated in triplicate for each plate. After the incubation, the plate wells were washed six times with phosphate buffer (provided in the kit), then 100 µL of the TMB One Component HRP microwell substrate (BioFX Laboratories Inc., Owings Mills, MD, USA) was added to each well, and the plates were incubated for 30 min at 37 °C. The reaction was stopped by the addition of 2 M sulfuric acid (provided in the kit), and the absorbance at 450 nm of each well was measured using a 96-well plate reader (VERSAmax™; Molecular Devices, LLC, San Jose, CA, USA). Protein concentrations were determined using the Pierce™ BCA Protein Assay Kit (Pierce Biotechnology, Thermo Fisher Scientific, Rockford, IL, USA). The SURASE B-96 quantitative standard panel is included with each assay.

Animal tests

The animal tests were conducted at Animal Technology Laboratories, Agriculture Technology Research Institute (Chunan, Taiwan). Specific pathogen-free (SPF) pigs, four males and three females, three weeks old, 19 kg of average body mass, were provided by the Cheng-Yu Pig Farm (Hsinchu City, Taiwan). The fruiting bodies of transgenic *F. velutipes* with the highest HBsAg content were used for the animal tests. Four pigs, two males and two females, in the experimental group were each fed with 9 g dry transgenic *F. velutipes* fruiting body powder every 3 days for 6 consecutive weeks, followed by feeding once a week for another 14 consecutive weeks. Another three pigs fed with wild-type *F. velutipes* were used as the control group. The fruiting body powder was mixed with 2 kg fodder composed of corn, soybean and rice bran (Cheng-Yu Pig Farm), and left in the cage for 24 h or until all food was consumed. Regular fodder was used throughout the experiment. Starting from the sixth week, blood samples were collected periodically for antibody assay.

Measurement of anti-HBsAg antibodies

HBsAg antibodies were detected with Elecsys anti-HBs (Roche, Basel, Switzerland) electrochemiluminescence immunoassay according to the manufacturer's instructions using Modular E170 immunology analyzer (Roche). Anti-HBsAg antibodies in the sample, biotinylated HBsAg, and HBsAg labelled with a ruthenium complex reacted to form a sandwich complex. After the addition of streptavidin-coated microparticles, the complex bound to the solid phase *via* interaction of biotin and streptavidin. The reaction mixture was aspirated into the measuring cell where the microparticles were magnetically captured onto the surface of the electrode. Application of a voltage to the electrode induced chemiluminescent emission which was measured by Modular E170 immunology analyzer (Roche). Results were determined *via* a calibration curve which was instrument-specifically generated by 2-point calibration and a master curve provided *via* the reagent barcode, calculated in milli-international units per volume (mIU/mL), and reported as positive when the value was more than 10 mIU/mL.

Statistical analysis

Least significant difference (LSD at $p=0.05$) test was used to identify significant differences of the expression level among three transformants. Significant difference was defined at $p<0.05$.

RESULTS AND DISCUSSION

F. velutipes transformation

A. tumefaciens containing p0390, p0390-AH or p0390--AH-FmHB was co-cultivated with *F. velutipes* MMPs in induction medium for 5–7 days and then transferred to selective agar plates containing 30 µg/mL hygromycin B. The plasmids p0390 and p0390-AH were used as negative and positive control, respectively. As shown in **Fig. 2**, hygromycin B-resistant mycelia appeared approx. 2-4 weeks after the transfer to the selective agar plates; however, *F. velutipes* MMPs co-cultivated with *A. tumefaciens* carrying the empty vector p0390 failed to grow. To confirm the stability of the putative transformants carrying the plasmid p0390-AH-FmHB, they were subcultured three times on the agar plates under selection pressure to ensure that the hygromycin B resistance gene and the *HBsAg* gene were stably expressed in *F. velutipes*. Even though the transformants can stably grow on the selective agar plates, their mycelia might be composed of both transgenic and non-transgenic cells. To resolve this problem, mushroom fruiting and basidiospore mating were conducted.

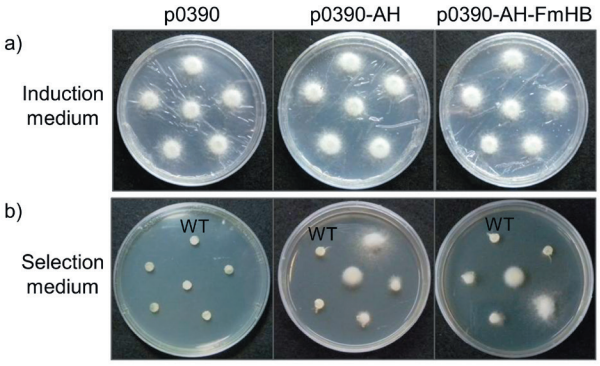

Fig. 2. The process of *Agrobacterium tumefaciens*-mediated transformation: a) *Flammulina velutipes* MMPs co-cultured with *A. tumefaciens* transformants (p0390, p0390-AH and p0390-AH-FmHB) on induction medium, b) co-cultured MMPs transferred onto selection medium with 30 μg/mL hygromycin B. WT=wild-type *F. velutipes*

Transformant fruiting and mating

Two putative transformants were selected as the parental strains for fruiting. The fruiting bodies of transformants 1 and 2 were cultivated and the basidiospores were collected. The basidiospores were separated by spreading them onto CYM agar plates containing 30 μg/mL hygromycin B to form monokaryotic mycelia. PCR analysis served to investigate the presence of the *HBsAg* gene. **Fig. 3** illustrates that *HBsAg* gene was stably maintained in the parental (**Fig. 3a**) and the filial generation (**Fig. 3b**) of dikaryotic transformants, and in the monokaryotic mycelium transformants derived from parental generation (**Fig. 3c**). The successful mating was checked by the clamp connection and by compatible growth of two monokaryotic mycelial plugs. No clamp connections were observed in the monokaryotic mycelia (**Fig. 4a**). Mating was conducted by placing two monokaryotic mycelial plugs on the same CYM agar plate containing 30 μg/mL hygromycin B and incubating at 23 °C for 3 weeks. The successful mating was

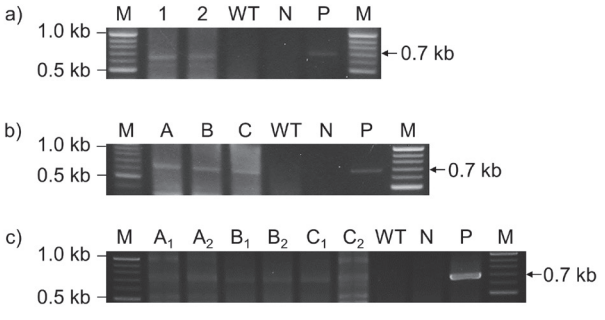

Fig. 3. PCR analysis of putative transformants: a) the parental generation of HBsAg *Flammulina velutipes* dikaryotic transformants, b) the filial generation of HBsAg *F. velutipes* dikaryotic transformants, and c) the monokaryotic mycelium transformants derived from parental generation. Lanes 1 and 2=the parental generation of HBsAg *F. velutipes* dikaryotic transformants, lanes A, B and C=the filial generation of HBsAg *F. velutipes* dikaryotic transformants, lanes A_1, A_2, B_1, B_2, C_1 and C_2=the mating type of monokaryotic transformants of A, B and C, lane WT=wild-type *F. velutipes*, lane P=positive control using plasmid p0390-AH-FmHB, lane N=negative control, and lane M=marker

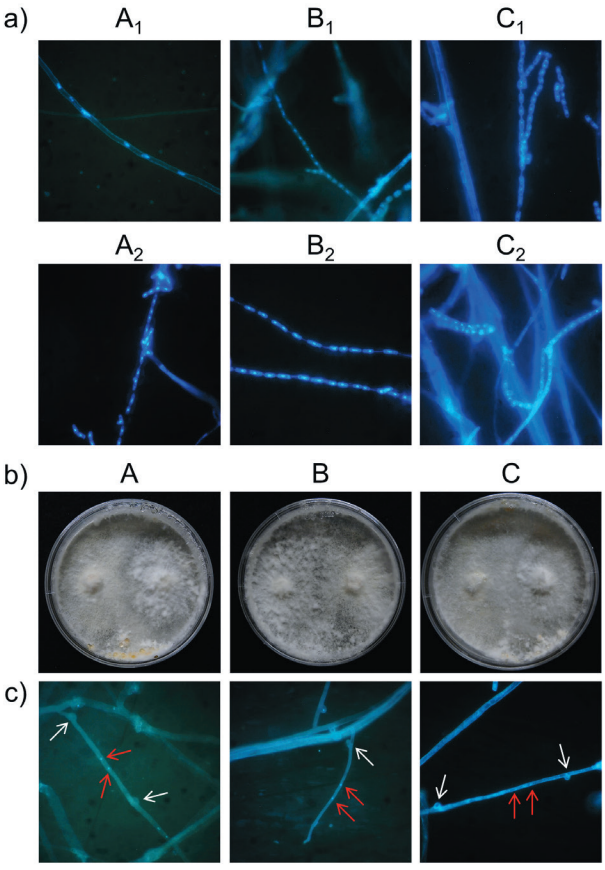

Fig. 4. The mating process of *Flammulina velutipes* transformants: a) microphotographs of monokaryotic mycelia of transformants stained by DAPI, b) morphology of the filial generation of HBsAg *F. velutipes* transformants after mating, and c) microphotographs of filial generation of HBsAg *F. velutipes* transformants stained by DAPI. Lanes A, B and C=the filial generation of HBsAg *F. velutipes* dikaryotic transformants, lanes A_1, A_2, B_1, B_2, C_1 and C_2=the mating type of monokaryotic transformants of A, B and C, white arrow=clamp connection, red arrow=nucleus

confirmed by compatible growth between two monokaryotic mycelial plugs (**Fig. 4b**) and clamp connections (**Fig. 4c**). These results show that both nuclei in each pure cell of the filial dikaryotic mycelia contained the target gene. The dikaryotic mycelia containing the *HBsAg* gene remained stable after repeated subculture (data not shown). The fruiting bodies of the stable dikaryotic mycelia were cultivated for further HBsAg analysis and for the animal tests.

HBsAg quantification and qualification via ELISA and western blot analysis

The western blot analysis of three HBsAg transgenic *F. velutipes* fruiting bodies is shown in **Fig. 5**. Immunoblotting with the monoclonal anti-HBsAg antibody detected a band at 25.4 kDa in the positive control (without the endoplasmic reticulum (ER) retention sequence) and a band at 26.1 kDa in the transformants, while no signal was observed in wild-type *F. velutipes*. The HBsAg expression level in the fruiting bodies of these three transformants was determined by ELISA (**Fig. 6**).

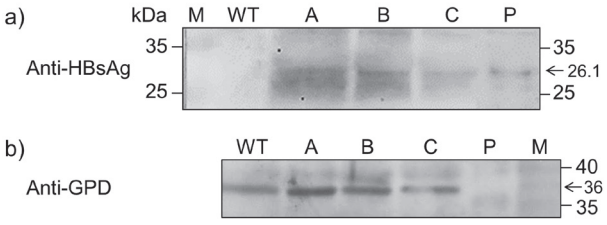

Fig. 5. Expression of HBsAg in *Flammulina velutipes* transformants: a) the western blot analysis of three HBsAg transgenic *F. velutipes* fruiting body immunoblotting with the monoclonal anti-HBsAg antibody, b) immunoblotting with the monoclonal anti-GPD antibody. GPD=glyceraldehyde-3-phosphate dehydrogenase. Lane WT=wild type, lanes A, B and C=the filial generation of HBsAg *F. velutipes* transformants, lane P=positive control, a commercial HBsAg (226 amino acids, 25.4 kDa); the HBsAg expressed in transformants is 232 amino acids, 26.1 kDa, and lane M=marker

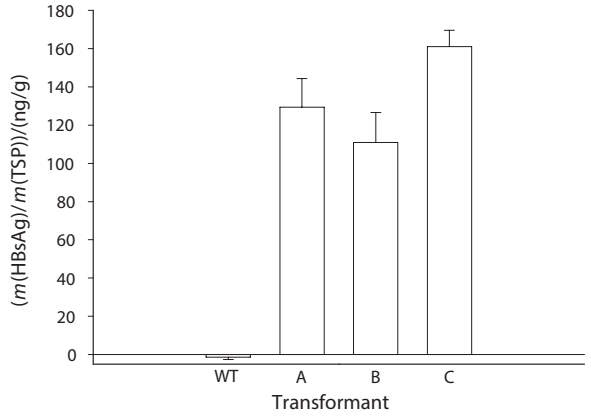

Fig. 6. HBsAg quantification of fruiting bodies for wild-type and transgenic *Flammulina velutipes*. WT=wild type, A, B and C=the filial generation of HBsAg *F. velutipes* transformants, TSP=total soluble protein. The HBsAg assays were repeated three times. The error bars indicate the standard deviation. Expression level among transformants A, B and C is not significantly different ($p>0.05$)

The HBsAg expression level of transformants A, B and C was (129.3±15.1), (110.9±1.7) and (161.1±8.5) ng/g TSP, respectively. Transformant C was further used for the large-scale production of fruiting bodies for animal tests. The results indicate that stable HBsAg transgenic *F. velutipes* fruiting bodies were generated using monokaryotic mycelium selection and the basidiospore mating.

Previous research has expressed HBsAg in edible plant tissues, including lettuce leaves (*23*), cherry tomatillos and tomatoes (*7*), at less than 0.000001 % of fresh mass, while in potato tubers it was up to 0.002 % of fresh mass (*5*). The low antigen expression level might be attributed to molecular biology mechanisms such as codon bias, improper mRNA splicing and post-translational modification, or to impure transgenic mycelia. In this study, the codons of HBsAg were optimized by checking the cDNA sequences *via* reversed transcription PCR, thus eliminating the potential for improper mRNA splicing. Since the stability of HBsAg virus-like particles relies on disulphide bond formation in the ER (*24*), an ER retention signal, HDEL, was constructed to ensure the retention of HBsAg in the ER and increase the likelihood of forming the correct structure. In addition, pure HBsAg transgenic *F. velutipes* mycelia were obtained *via* a mating scheme. The low expression level may be a result of incomplete protein extraction and weak antibody recognition since the antigenic domain presented on hepatitis B virus envelope is a conformational rather than a linear epitope (*25*). The development of more efficient HBsAg extraction and detection methods from transgenic *F. velutipes* fruiting bodies is required.

Oral immunogenicity of HBsAg was stimulated by transgenic F. velutipes

The immune response to the HBsAg transgenic *F. velutipes* fruiting bodies in pigs is shown in **Table 1**. No anti-HBsAg antibody was detected in the control group throughout the experiment. The blood sample collected from male pig 15903 in the experimental group displayed an antibody response value of 5.36 mIU/mL in week 10, which is below the positive response value (10 mIU/mL). The HBsAg-specific IgG level in this pig reached a maximum of 14.9 mIU/mL in week 14 and then decreased thereafter. Female pig 17215 had HBsAg levels of 9.75, 17.86 and 39.87 mIU/mL in weeks 14, 18 and 20, respectively. The other two pigs in the experimental group did not produce detectable antibodies. These results provide solid evidence that a primary anti-HBsAg immune response can be stimulated in pigs by oral administration of transgenic *F. velutipes* fruiting bodies.

Table 1. Serum anti-HBsAg antibodies detected in pig

Group	Pig code (gender)	*t*/week					
		6	8	10	14	18	20
		N(antibody)/(mIU/mL)					
Control	16113 (F)	2.00	2.00	2.00	2.00	2.00	2.00
	17202 (M)	2.00	2.00	2.00	2.00	2.00	2.00
	17204 (M)	2.00	2.00	2.00	2.00	2.00	2.00
Experiment	16114 (F)	2.00	2.00	2.00	2.00	2.00	2.00
	17215 (F)	2.00	2.00	2.00	9.75	17.86	39.87
	15903 (M)	2.00	2.00	5.36	14.90	2.00	2.00
	17308 (M)	2.00	2.00	2.00	2.00	2.00	2.00

mIU=milli-international units, F=female, M=male
Due to severe weather conditions no blood was collected in the 12th and 16th week

Several studies have suggested that increased immunogenicity of HBsAg using oral administration *via* transgenic potatoes and tomatoes usually requires an adjuvant, such as cholera toxin (CT). Richter *et al*. (*5*) reported that feeding mice with 5 μg HBsAg expressed in potato tuber and 10 μg CT prompted a serum response peaking at approx. 70 mIU/mL. In the absence of adjuvant, primary immunization with 40 μg oral doses of potato-expressed antigen did not induce a response (*6*), but otherwise with 60 μg oral doses antigen prompted an antibody response up to 170 mIU/mL (*26*). In this study, a positive response was obtained by feeding transgenic *F. velutipes* fruiting bodies without CT, indicating that the mushroom polysaccharides might serve the role of an adjuvant. This observation is consistent with the report of Ichinohe *et al.* (*14*), who demonstrated the effectiveness of mycelial extracts derived from edible mushrooms as adjuvants for an intranasal influenza vaccine.

CONCLUSION

In this study, we obtained stable HBsAg transgenic *F. velutipes* dikaryotic mycelia *via* a mating scheme and confirmed that the production of anti-HBsAg antibodies was stimulated in pigs by feeding them *F. velutipes* fruiting bodies, demonstrating the potential of the application of *F. velutipes* in oral vaccines. This is the first report to show that the immunogenicity in pigs can be achieved by feeding them transgenic HBsAg mushroom.

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CONFLICT OF INTEREST

LHH is a current employee, and YTL and CLG are former employees of MycoMagic Biotechnology Co., Ltd. HYL is a former graduate student advised by CTH at National Taiwan University. CTH is bound with a Technology Transfer Agreement between MycoMagic and National Taiwan University (09T--101203-1N-A). The authors have declared that there are no other competing interests.

ETHICS APPROVAL

All animal tests were conducted by the Animal Technology Laboratories, Agriculture Technology Research Institute (Contract No.: BL-11-T020801-P) and were in compliance with Laboratory Animal Welfare and Ethics guidelines as issued by the Council of Agriculture, Taiwan. The protocol was approved by the Committee on Laboratory Animal Management of Agriculture Technology Research Institute, Taiwan.

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